Vol. 89, No. 3 December, 1945

THE
BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

NATURAL HETEROAGGLUTININS IN THE SERUM OF THE SPINY LOBSTER, PANULIRUS INTERRUPTUS. II. CHEMICAL AND ANTIGENIC RELATION TO BLOOD PROTEINS[1]

ALBERT TYLER AND BRADLEY T. SCHEER

William G. Kerckhoff Laboratories of the Biological Sciences, California Institute of Technology, Pasadena

In a previous report (Tyler and Metz, 1945) it has been shown that lobster-serum contains at least ten heteroagglutinins for sperm or blood cells of various animals. Each of the heteroagglutinins was found to act on all the species tested that belong to the same group of animals. Since the group, in most instances, represents a taxonomic class, the heteroagglutinins are termed class-specific. The heteroagglutinins were found to be most probably protein, and by means of electrophoresis they were shown to be distinct from the hemocyanin which Allison and Cole (1940) and Clark and Burnet (1942) had considered to be the sole protein present in lobster-serum.

The relatively small amount found to be present accounts for Allison and Cole's conclusion which was based on approximate identity of the copper to protein nitrogen ratios of purified hemocyanin and of whole serum. Clark and Burnet's evidence was actually to the effect that there is no protein present with active antigenic properties different from that of pure hemocyanin. This is in accord with the results obtained with antisera prepared against heteroagglutinin by injecting rabbits with agglutinin that had been absorbed on rabbit cells. In the present paper a precipitation method for preparing the heteroagglutinins free of hemocyanin is described, and results of an electrophoretic examination of the material are presented. The agglutinating action of fibrinogen preparations from plasma and further serological tests are also reported.

Material and Methods

Blood is quite easily obtainable from lobsters by means of a syringe inserted, between cephalothorax and abdomen, into the pericardial chamber. A twelve-inch lobster yields, in this manner, about 20 to 30 ml. of blood. For serum the blood was generally defibrinated by shaking with glass beads, filtered, and centrifuged; or it was occasionally allowed to clot, forced through a fine mesh wire screen, and centrifuged. For plasma the blood was drawn into a small amount of sodium

[1] This work has been aided by grants from the American Philosophical Society and the Rockefeller Foundation.

citrate solution, then subsequently filtered, centrifuged, and dialyzed against saline. One volume of 10 per cent citrate suffices to prevent fibrin-clotting in about 30 volumes of blood.

The agglutinative tests were made as previously described (Tyler and Metz, 1945) by mixing equal volumes of the sperm or blood cells (of sea-urchin, sheep, or other animal) and of serial two-fold dilutions of the test-solution adjusted to the appropriate salinity. Deviations from these proportions are specified in the tests.

Experimental Part

Separation of heteroagglutinins from hemocyanin by isoelectric precipitation

Hemocyanin was prepared from serum by isoelectric precipitation essentially as described by Allison and Cole (1940) and by Rawlinson (1940). This consists in dialysis against distilled water and then against dilute acetate buffer at the pH of the isoelectric point. Further purification is obtained by repeated solution in dilute ammonia and reprecipitation, by addition of acetate buffer (0.1 M., pH 4.5).

Rawlinson (1940), in the course of purification of hemocyanin from the *plasma* of the Australian spiny lobster, noted the presence of small amounts of protein which he considered to be fibrinogen. Such a non-hemocyanin protein is obtainable from the *serum* of the California spiny lobster, *Panulirus interruptus*.

When samples of serum or plasma of *Panulirus* were dialyzed against dilute, pH 4.5, acetate buffer, there invariably appeared small amounts of a pale precipitate that separated before the hemocyanin started to come down. The precipitates ranged in color from white to pink. After centrifugation, washing with distilled water and solution in dilute ammonia, the material was reprecipitated by slow addition of 0.01 M., pH 4.5 acetate buffer. The material was regularly found to start to precipitate at pH 5.0 and reach a maximum at pH 4.8. From the supernatants of the first precipitates the blue-colored hemocyanin was precipitated by continuation of the dialysis against the pH 4.5 buffer. The hemocyanin was obtained in crystalline form from concentrated solutions of it in dilute ammonia by the slow addition of dilute acetate buffer. Its precipitation was found to begin at pH 4.6 and to be complete at 4.5 to 4.4.

Samples of the purified hemocyanin and of the pale precipitate were tested for their ability to agglutinate the sperm or blood cells of various animals. After adjustment of the solution to appropriate pH and salinity by dialysis, they were tested on one per cent suspensions of the sperm of the polychaet, *Chaetopterus variopedatus;* the sea cucumber, *Stichopus californicus;* the starfish, *Pisaster ochraceus*; the sea-urchin, *Strongylocentrotus purpuratus*; the sea-squirt, *Ciona intestinalis;* and the grunion (smelt), *Leuristhes tenuis;* and of the erythrocytes of the sand bass, *Paralabrax maculatofasciatus;* the frog, *Rana pipiens;* the chuckwalla, *Sauromalus ater;* the pigeon, and sheep. The hemocyanin preparations, containing this material in amounts as great as or greater than normally present in the serum, were found to be completely inactive. The preparations of the pale, pH 4.8-5.0, precipitate gave very good agglutination of the cells of all the above named species.

Titer determinations were made with one of these preparations on sperm of *Strongylocentrotus*. In this case 0.2 ml. of serial two-fold dilutions of the solution were mixed with one drop of 10 per cent sperm-suspension. The protein con-

centration (from Kjeldahl nitrogen determination) of the solution was 0.7 per cent and its titer (minimum dilution giving definite microscopic agglutination) was 128. A sample of serum containing 5 per cent protein gave at the same time a titer of 256. This preparation showed, then, about 3½ times the activity of the whole serum.

Electrophoretic examination of the pale precipitate [2]

Another sample of the material freed of hemocyanin was reprecipitated at pH 5, dissolved in dilute ammonia, and dialyzed for 2 days in the cold against barbiturate buffer ($\mu = 0.05$) at pH 7.7. It was then examined electrophoretically in the

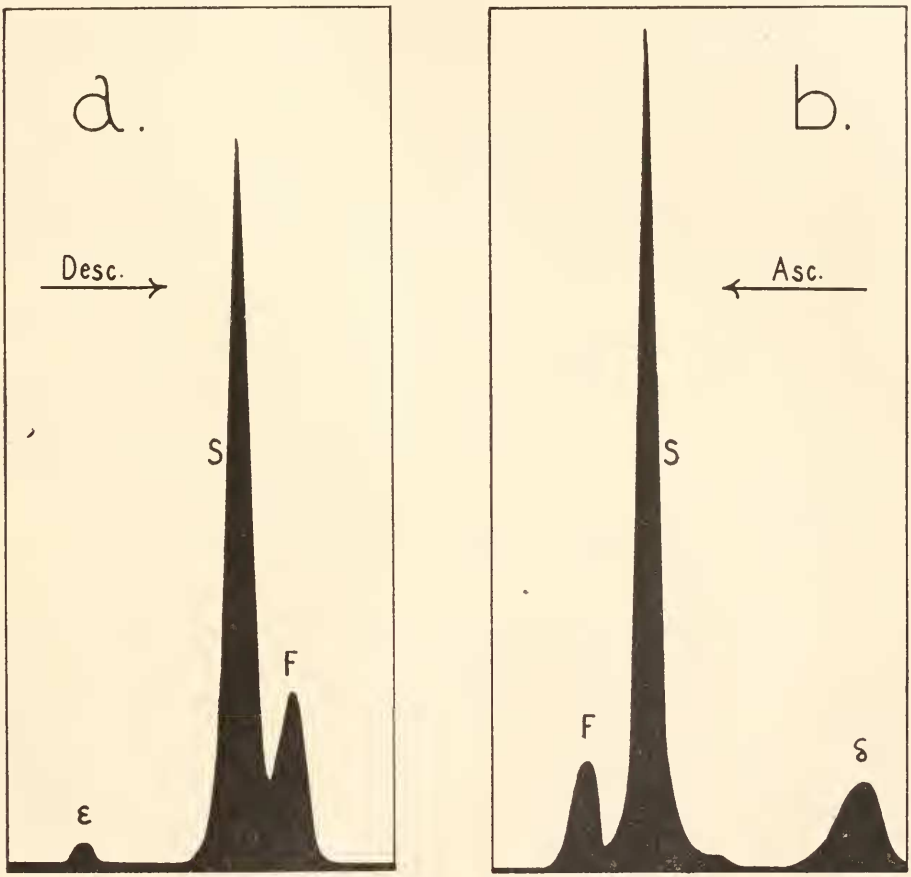

FIGURE 1. Electrophoretic patterns of pale (pH 5) precipitate from lobster-serum. *a*, descending (desc.) side; b, ascending (asc.) side; after 59 minutes of electrophoresis at pH 7.7, ionic strength 0.05 and 14.8 ma. Arrows show direction of migration. See text for further description.

[2] The apparatus employed was that constructed in the Division of Chemistry by Dr. Stanley M. Swingle to whom we are indebted for the electrophoresis of this material.

Tiselius' (1937) apparatus using the scanning method of Longsworth (1939). After 59 minutes of electrophoresis with a current of 14.8 ma., the patterns shown in Figure 1 were obtained. As may be seen from the figure two components, besides the δ- or ε-boundaries, are present in the serum. From the relative areas covered by the peaks the ratio of amount of slow component to that of fast component is approximately 5:1. At the end of the run the fast moving component was removed from the ascending side and the slow component (plus δ), from the descending side of the electrophoresis cell. After dialysis against normal saline, determinations were made of their agglutinative titers for rabbit cells and of the Kjeldahl nitrogen content. Samples of the original solution of the pale precipitate (taken from the cell after the run) and of normal lobster-serum were tested at the same time. The results are given in Table I. The nitrogen content of the solutions does not represent the relative concentrations of the components present in the original solution since there was some dilution with buffer upon their removal from the electrophoresis cell. As may be seen in Table I, the solution of the fast component showed no agglutinative activity for rabbit-erythrocytes although its nitrogen content was about one-third that of the slow component. The slow component proved highly active, giving almost twice the titer (per mg. N. content of solution) of the original solution and 24 times that of whole serum. This is approximately the order of magnitude of activity obtained (Tyler and Metz, 1945) for the components isolated by electrophoresis from whole serum.

TABLE I

Agglutinative titers of components obtained by electrophoresis of the pale (pH 5) precipitate from lobster-serum

Material	mg. Kjeldahl N. per ml.	Agglutinative titer on 1% rabbit cells	Titer/mg. N.
Fast component (F of Figure 1)	0.29	0	0
Slow component (S of Figure 1)	1.008	128	128
Original solution (from cell-residue)	3.85	256	66.5
Whole serum	11.97	64	5.3

The slow component obtained here was also tested on cells of all the animals listed on page 194, with the exception of *Sauromalus* and *Leuristhes*. It proved to be highly active with all of them. In the previous report lobster-serum was shown to contain at least ten "class-specific" heteroagglutinins. It is evident from the present results that these are represented by a single electrophoretic component of the serum, unless there is some active component in the stationary δ- or ε-boundary. The latter is, however, highly unlikely since the original material for the present test was obtained by precipitation at pH 4.8 to 5.0 and the electrophoresis was run at pH 7.7. For any material to remain in these stationary boundaries it would have to be isoelectric at the latter pH.

Preparation of fibrinogen and tests for heteroagglutinating activity

Lobster-plasma upon being brought to 25 per cent saturation with ammonium sulfate formed a white to pink precipitate which separated easily upon centrifuga-

tion. The precipitate was washed with distilled water and dissolved in sea water. Addition of fresh lobster-blood-cells to the solution caused it to form a firm clot. A pH 5.0 precipitate obtained directly from plasma was found to contain fibrinogen, which could be separated from the remaining protein material by precipitation with ammonium sulfate. None of the prepartions from serum were found to contain fibrinogen.

TABLE II

Agglutinative titers of protein preparations from plasma and serum

Material	mg. Kjeldahl N. per ml.	Agglutinative titer on *Strongylocentrotus* sperm	Titer per mg. N.
Fibrinogen preparation (I)	1.25	32 to 64	26 to 51
Pale precipitate (II)	1.25	64 to 128	51 to 102
Hemocyanin	7.4	0	0
Whole serum	8.5	128 to 256	15 to 30
Plasma	8.5	256 to 512	30 to 60

A fibrinogen preparation (I) was obtained from whole plasma by 25 per cent saturation with ammonium sulfate. The precipitate was dissolved and reprecipitated by dialysis to pH 5.0. This preparation was tested for agglutinating action on sperm of *Strongylocentrotus* in the same manner as on page 194. The supernatant from the 25 per cent ammonium sulfate precipitate was dialyzed against tap water and then brought to approximately pH 5 by dialysis against pH 4.5 buffer. This gave a pale precipitate (II) which resembled the pale precipitate from serum. After solution and dialysis against sea water it, too, was tested for agglutinating activity. The results are given in Table II along with simultaneous tests of whole serum, plasma, and hemocyanin. The presence of calcium in the sperm suspension does not interfere with the tests, since clotting of the fibrinogen does not occur unless fresh lobster-blood-cells are added. As the table shows, plasma has about twice the agglutinating activity of serum. The fibrinogen preparation proved about half as active as the pale precipitate.

Another pale precipitate was also obtained directly from plasma by dialysis against pH 4.5 buffer. When the precipitate was dissolved and brought to 25 per cent saturation with ammonium sulfate there separated out some material that proved to be fibrinogen. It appears from the experiments reported above that the isoelectric point of fibrinogen is not greatly different from that of the heteroagglutinin found in serum. This conclusion was verified by Mr. Maurice Rapport, who repeated some of our experiments, and made an electrophoretic examination of plasma and of protein preparations separated from plasma. The pH 5.0 precipitate from plasma showed two electrophoretic components, the patterns being similar to those of Figure 1. The smaller, faster component probably corresponded to the fast component observed in serum preparations. The other component, containing agglutinating activity, could not be separated further during 100 minutes of electrophoresis at pH 7.3, 1.2° C. and 20 ma.

Precipitation of the pH 5.0 precipitate from plasma with ammonium sulfate at 40 per cent of saturation removed nearly all of the agglutinating activity, but left behind a small amount of protein material. The ammonium sulfate precipitate

contained 3.5 mg. Kjeldahl N./ml, and had a titer of 64 against *Strongylocentrotus* sperm. The supernatant contained 1.6 mg. N./ml, and titrated only to 4. Mr. Rapport showed that this small residue migrated rapidly in the electrophoresis apparatus at pH 7.3. It probably corresponded to the fast component from serum.

In the absence of more exhaustive chemical and electrophoretic separations it is not possible to decide with certainty whether the agglutinative activity found in fibrinogen preparations is associated with fibrinogen itself, or is due to the presence in these preparations of the heteroagglutinin fraction which is present in serum.

Antigenic relationship of the blood proteins

Two rabbits that were each given two courses of intravenous and intra-abdominal injections with a total of 375 mg. of purified hemocyanin produced very good precipitating antisera. The titers (end point of precipitation on mixing equal volumes of antiserum and serial dilutions of a 10 per cent hemocyanin solution) ranged from 10,000 to 20,000 in terms of antigen dilution and optimal proportions (second optimum, see below) were obtained at approximately one volume of 10 per cent hemocyanin to 10 to 20 volumes of antiserum. The antisera also reacted very well with whole lobster-serum, the optimal proportions point being about 9 volumes of antiserum to one volume of the lobster-serum.

Tests were then made of the ability of antiserum vs. hemocyanin to remove natural heteroagglutinin from whole lobster-serum. One volume of lobster-serum was absorbed with 9 volumes of the rabbit antiserum and the supernatant tested for ability to agglutinate rabbit-erythrocytes and *Strongylocentrotus* sperm. The absorbed serum gave no reaction with these cells, while control lobster-serum gave good agglutination out to dilutions of 1/90 (+ + + reaction) with the rabbit cells and 1/80 (+ reaction) with the *Strongylocentrotus* cells respectively.

It appears, then, that antibodies prepared against hemocyanin also react with the natural heteroagglutinins present in lobster-serum.

One of the antihemocyanin rabbit sera was also titrated with the solution of electrophoretically purified heteroagglutinin (slow component). A titer (dilution of antigen) of 128 was obtained for this solution which contained one mg. Kjeldahl N. per ml. A control hemocyanin solution containing 8 mg. N. gave a minimum titer (end point not reached) of 4096, or 512 per mg. N.

Another antiserum against hemocyanin was also titrated with various protein fractions separated from lobster-blood. The titer (dilution of antigen) of reprecipitated hemocyanin was 20,000 for a solution containing 6.6 mg. Kjeldahl N. per ml. or 3000 per mg. N. For the heteroagglutinin (pH 5 precipitate from serum, reprecipitated), the titer was 200 for a solution containing 1.6 mg. N. per ml. or 125 per mg. N. For the fibrinogen (ammonium sulfate precipitate from plasma), the titer was 200 for 3.4 mg. N. per ml. or 60 per mg. N.

In these titrations, it was sometimes noted that precipitation occurred in the first few tubes, containing concentrated antigen solutions. In intermediate dilutions, no precipitation occurred, but a second zone of precipitation appeared in the higher dilutions. This was noted both with hemocyanin and fibrinogen, but not with the agglutinin preparation (pale precipitate from serum) used. Boyden and deFalco (1943) reported a similar double zone phenomenon with *Homarus* serum titrated against anti-*Homarus*-hemocyanin. They pointed out that this is indicative of the

presence of two kinds of antibodies in the antisera. However, this does not seem to be the entire explanation, since we find that absorption of a sample of antiserum with an amount of hemocyanin which corresponds to the lower of the two optima removes all antibody for the homologous antigen, as well as for fibrinogen and pale precipitate.

Two rabbits were also immunized with whole lobster-serum, each receiving a total of 5.5 ml. of serum in two courses of three injections each, with three weeks rest between courses. The antisera obtained one week after the last injection gave very good precipitation with the homologous antigen, optimal proportions (second optimum) being obtained with one volume of lobster-serum to approximately 16 volumes of antiserum. A sample was absorbed with purified hemocyanin and tested on whole serum, a concentrated solution of the pale (pH 5) precipitate, and a fibrinogen preparation. It failed to give precipitation with any of these antigens. This confirms the findings of Clark and Burnet (1942) and indicates that the other blood proteins have no active antigenic groups other than those present in the hemocyanin. Alternatively, the results might be explained on the basis of competition of antigens (see Sachs, 1929), such that the rabbit does not form antibodies against other antigens when one powerful antigen (the hemocyanin) is present in excess in the material (whole lobster-serum) used for immunization. However, in view of the analogous results obtained (Tyler and Metz, 1945) with antisera prepared against heteroagglutinin, and with antihemocyanin sera (above), the alternate explanation seems highly unlikely.

Summary

1. Lobster-serum contains small amounts of other protein constituents besides hemocyanin.

2. The "class-specific" heteroagglutinins of lobster-serum are found to reside in a component that is obtained free of hemocyanin by isoelectric precipitation at pH 4.8 to 5.0.

3. Electrophoresis of this "pale precipitate" reveals the presence of two components, of which the more slowly migrating one bears the heteroagglutinating activity. The ten separate "class-specific" heteroagglutinins are thus evidently represented by a single electrophoretic component.

4. There is some indication that fibrinogen obtained from the lobster plasma may also act as heteroagglutinin.

5. Antibodies produced in rabbits against purified hemocyanin also react with the slow electrophoretic component (heteroagglutinin) of the pale precipitate and with fibrinogen. Absorption tests with antisera vs. whole lobster-serum fail to reveal the presence of any specific antigenic groups other than those of the hemocyanin. The other blood proteins are, then, evidently serologically equivalent to hemocyanin.

LITERATURE CITED

Allison, J. B., and W. H. Cole, 1940. The nitrogen, copper and hemocyanin content of the sera of several arthropods. *Jour. Biol. Chem.*, **135**: 259–265.

Clark, Ellen and F. M. Burnet, 1942. The application of the serological methods to the study of crustacea. *Austral. Jour. Exp. Biol. and Med. Sci.*, **20**: 89–95.

Boyden, A., and R. J. DeFalco, 1943. Report on the use of the photronreflectometer in serological comparisons. *Physiol. Zool.*, **16**: 229–241.

Longsworth, L. G., 1939. A modification of the Schlieren method for use in electrophoretic analysis. *Jour. Amer. Chem. Soc.*, **61**: 529–530.

Rawlinson, W. A., 1940. Crystalline haemocyanin: some physical and chemical constants. *Austral. Jour. Exp. Biol. and Med. Sci.*, **18**: 131–140.

Sachs, H., 1929. Antigene und Antikörper (c) die Konkurrenz der Antigene. *Handbuch der Norm. und Path. Physiol.*, **13**: 444–446.

Tiselius, A., 1937. A new apparatus for electrophoretic analysis of colloidal mixtures. *Trans. Faraday Soc.*, **33**: 524–531.

Tyler, A., and C. B. Metz, 1945. Natural heteroagglutinins in the serum of the spiny lobster, Panulirus interruptus. I. Taxonomic range of activity, electrophoretic and immunizing properties. *Jour. Exper. Zool.*, in press.